PATENT
P-4739-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Moutsatsos et al.	Examiner:	Sandals W.
Serial No.:	09/148,234	Group Art Unit:	1636
Filed:	September 4, 1998		
Title:	GENETICALLY ENGINEERED CELLS WHICH EXPRESS BONE MORPHOGENIC PROTEINS		

DECLARATION UNDER RULE 37 C.F.R. 1.132

Assistant Commissioner for Patents
Washington, DC 20231

I, Edward Schwarz, Ph.D., a citizen of the United States of America, residing at 125 Barclay Square Drive, Rochester, New York, 14618, USA, hereby declare:

1. I am a Professor of Orthopedics, Medicine, Biomedical Engineering, Pathology, Microbiology and Immunology at the University of Rochester, Rochester, New York, USA. I have a Ph.D. in Microbiology and Immunology from the Sue Golding Graduate Division of the Albert Einstein College of Medicine, Bronx, New York. My fields of expertise are Molecular Biology, Gene Therapy, Stem Cell Biology and Musculoskeletal Tissue Engineering. Specifically, I have been involved in the study of revitalizing bone grafts using gene therapy and stem cell approaches.

2. My Curriculum Vitae and list of publications are attached herewith as Appendix 1.

3. I have read the subject Application and have reviewed the patent Prosecution History, including the Office Action of April 04, 2006, November 15, 2005, July 21, 2005, December 7, 2004, October 22, 2004, June 21, 2004, and September 9, 2003. The subject Application describes *inter alia, ex-vivo* methods of transforming or transducing mesenchymal stem cells with a nucleic acid, which encodes for BMP-2 protein, for implantation in a subject in need of bone repair or regeneration.

4. Claim 24 of the subject Application recites a method of inducing organized, functional bone formation at a site of bone infirmity in a human, comprising the steps of:

(a) transforming a cultured mesenchymal stem cell with a DNA encoding human bone morphogenesis protein 2 (BMP-2);

(b) culturing the cultured mesenchymal stem cell transformed in step (a), under conditions enabling expression of said DNA encoding bone morphogenesis protein 2; and

(c) implanting said cultured mesenchymal stem cell in an allogeneic subject, at a site of bone infirmity

whereby autocrine and paracrine effects of expressed human bone morphogenesis protein 2 at said site of bone infirmity result in organized, functional bone formation, thereby inducing organized, functional bone formation at a site of bone infirmity.

5. The specification provides exemplification of the claimed material, whereby mesenchymal stem cells transduced with a BMP-2 containing construct effectively stimulated functional bone formation, including formation specifically along defect edges. Example 1 demonstrated that regulated expression of BMP-2 was highly effective in promoting bone formation at a segmental defect site. Example 4 demonstrated that marrow osteoprogenitor cells genetically modified to express BMP were effective in promoting bone

formation as well. Example 8 demonstrated the advantage of combined paracrine and autocrine effects afforded by the use of the ex-vivo transformed cells, which resulted in superior bone formation, when compared to paracrine effects alone, and Example 11 demonstrated that only MSC expressing BMP-2 (MSC-BMP-2) provided was incorporated in newly formed bone trabecules, and formed superior quantitative and qualitative bone, this despite the fact BMP-2 was secreted at a roughly 100 times lower concentration than that of CHO cells transduced to express BMP-2 and 100 times lower concentration than the amount of BMP-2 loaded on collagen sponges.

6. The Examiner rejected the claims of the above-identified application as allegedly being obvious to one skilled in the art, based on Ahrens et al. (DNA and Cell Biology, Volume 12, NO. 10, pages 871-880, 1993) and in view of United States Patent No. 5,763,416 (Bonadio et al.) and United States Patent No. 6,048,964 (Lee et al.). As I understand, the Examiner alleged that Bonadio's described targeting of progenitor cells *in vivo*, combined with Ahren's described transduced cell render the claims obvious to one of ordinary skill in the art. The Examiner rejected claim 27 as allegedly being obvious to one skilled in the art in view of the above cited references, further in view of Wozney, and claim 28 as allegedly being obvious to one skilled in the art in view of the above cited references, further in view of Hattersley.

7. It is my opinion that the Examiner is incorrect in his assertion. In my opinion, Bonadio does not provide a credible foundation for a method of stimulating bone formation at a site of a bone infirmity by implanting a mesenchymal stem cell transformed/transduced with a BMP-2 construct. Bonadio targets a heterogeneous population of cells. While Bonadio describes specific targeting of progenitor cells, this contention is not credible, since stem cells if present, are in negligible amount, at the site of gene transfer. Cellular uptake of DNA is a complex process, and varies in terms of the different cell types and/or stage of differentiation of such cells, and/or the efficiency or even plausibility of such uptake. The type of vector employed will also necessarily affect the kinetics of such uptake.

8. Bonadio used a gene transfer system, which is not suitable for transfection of undifferentiated cells. For example, the adenoviral vectors used by Bonadio

depend upon CAR-mediated uptake, a receptor known to not readily be expressed on immature, noncommitted progenitor cells (see for example, Rebel V.I. et al., Stem Cells (2000) 18: 176-82; Zhao Q. et al., Blood (1994) 84:3660-6), at the time the invention was made. Therefore, based on my 18 years of experience and expertise in the field of Molecular and Cell Biology it is not credible, that at the time the invention was made, direct gene transfer experiments conducted by Bonadio targeted undifferentiated cells. Therefore, in my opinion Bonadio does not provide a foundation that BMP gene transfer supplies more than paracrine effects for healing a bone infirmity and Bonadio cannot predict the organized functional bone formation of the instant invention which occurs as a consequence of transfer of an enriched population of ex-vivo cultured BMP-2 expressing MSC (MSC-BMP-2).

9. The experiments in examples 7, 9, and 11 conducted with CHO cells expressing BMP-2, and collagen sponges loaded with purified BMP-2 can serve as an indication of the contrast between what Bonadio describes and the instant invention. Differentiated cells at a site of bone infirmity are the bulk recipients of the gene construct of Bonadio, or responders to the purified protein produced at the site, and not stem or progenitor cells. Such cells did not home to the site of bone infirmity, and did not produce as qualitatively or quantitatively organized functional bone, as compared to the MSC-BMP-2. The CHO-BMP and loaded sponge controls serve as reliable indicators as to what direct gene transfer produces at a fracture site.

10. Lee describes osteogenesis via local administration of a morphogenic protein. Osteogenesis is assumed in Lee, based solely on alkaline phosphatase production by osteoblasts in culture. Lee does not provide any demonstration of bone formation, but rather production of alkaline phosphatase alone, *in vitro*. There is no indication, based on Lee, that ex-vivo cultured BMP-2 transduced MSC promote organized functional bone formation *in vivo*. Moreover, Lee's findings rely solely on paracrine effects of the BMP for stimulating osteoblast AP production. In my opinion, the data presented in the subject Application demonstrate that paracrine effects alone do not result in organized, functional bone formation.

11. Ahrens discloses *in vitro* responses of progenitor cells to a group of osteoinductive compounds (which include, *inter-alia*, a BMP). Ahrens provides no foundation for the likelihood that implantation of such cells, transduced only with a vector expressing a BMP, *in vivo*, will stimulate organized, functional bone formation at a site of bone infirmity. Such a result is predicated on appropriate cell homing and orientation along the defect edges, a result, which could not have been foreseen, based on Ahrens.

12. Neither Ahrens nor Lee describe, or provide a foundation for cells alone, *in lieu* of any other osteoinductive matrix, stimulating bone formation. Certainly neither describe nor provide any foundation for organized, functional bone formation of the instant invention, i.e. bone formation along fracture defect edges, as demonstrated in the subject Application. Accordingly, the differences in implantation of an enriched MSC population expressing BMP-2 promoting organized bone formation, within the boundaries of the fracture edges, and lack of appreciable bone resorption could not have been predicted, based on Bonadio, Ahrens, or Lee, alone or in combination.

13. The Examiner cited the Fang reference in support of Bonadio's mechanism being the same as that of the claimed invention. In my opinion, this is incorrect. Fang does not and cannot support this position for the following reasons:

    - Fang describes BMP-4 uptake by fibroblasts;
    - Since the BMP-4 uptake is by fibroblasts, its effect on bone formation must necessarily be via a paracrine mechanism;
    - The claimed invention, however, is directed to use of an ex-vivo cultured BMP-2 transformed/transduced progenitor cell, which exerts both paracrine and autocrine effects. The subject Application demonstrated that paracrine effects alone are insufficient to promote organized bone formation. Thus, Bonadio, even in view of Fang, does not support or provide a foundation for the claimed invention.

14. Moreover, Fang in fact contradicts Bonadio's contention that the mechanism of gene transfer results in the specific targeting of progenitor cells, since in Fang,

the construct was expressed by fibroblasts. Thus, Fang questions the credibility of Bonadio as to the mechanism of action of direct gene transfer in inducing bone formation.

15. Paracrine effects of BMP-2 are not sufficient to promote organized bone formation and prevent bone resorption at the site of a bone infirmity. Example 11 specifically demonstrates that better bone formation occurs when BMP is expressed predominantly by the MSCs as this provides for autocrine and paracrine effects, which yields better, qualitative and quantitative bone formation, which is organized along the defect edges, and subject to no appreciable resorption.

16. Wozney describes expression of a BMP2 receptor. However, no description of the use of *ex-vivo* cultured MSC transduced/transformed with BMP-2, in inducing organized functional bone formation at a site of bone infirmity in cells responding to the growth factor is disclosed in Wozney, Bonadio, Ahrens, or Lee, alone or in combination. In my opinion, therefore, Wozney's findings, when combined with the other cited references, neither implies, nor renders obvious the paracrine and autocrine effects on bone formation by implantation of ex-vivo MSC transduced/transformed with BMP-2, further comprising a BMP-2 receptor.

17. Hattersley describes the use of PTH and its receptor in combination with a BMP, for applications in tissue repair. Hattersley neither alone, nor in combination with the above cited references describes, nor provides any foundation for the use of *ex-vivo* cultured MSC transduced/transformed with BMP-2, with or without further expression of PTH and its receptor in inducing organized functional bone formation at a site of bone infirmity.

18. Accordingly, it is my opinion that none of the cited references, alone or in combination, describe, or provide a foundation for inducing enhanced, organized, functional bone formation at a site of bone infirmity in a human by implanting in an allogeneic subject, an *ex-vivo* cultured MSC transduced/transformed with a human BMP-2. The Bonadio, Ahrens, and Lee

disclosures do not credibly lead one to a population of cells capable of forming organized, functional bone at a site of bone infirmity, since it is improbable that Bonadio targets the cultured population, and further combination with Lee or Ahrens *in vitro* results do not support bone formation, in particular in the absence of exogenous provision of an osteoinductive matrix or compound.

19. The combination of Wozney, Hattersley, Fang, Bonadio, Ahrens, and Lee could not have predicted the unexpected results obtained in the claimed invention, which resulted in enhanced, organized, functional bone formation at a site of bone infirmity. *In-vivo* studies demonstrated that engineered progenitor cells (C3H-BMP2), in comparison to administration of 3 μg recombinant human BMP2, or engineered non progenitor cells (CHO-BMP2) produced enhanced bone formation, and most surprisingly, that the formation was in alignment with the original defect edge, this despite the fact that greater amounts of BMP-2 were secreted from the CHO BMP-2 cells.

20. The paracrine effects of BMP-2, as described in Bonadio and Fang, are not sufficient to promote organized bone formation and prevent bone resorption at the site of a bone infirmity. Nor does the combination of Bonadio, Fang, Ahrens, Wozney, Hattersley, or Lee lead one to the unexpected finding that an enriched population of MSCs expressing BMP-2 are particularly useful in promoting organized functional bone, by a process mimicking that which occurs in spontaneously healing bones, and producing qualitatively and quantitatively better bone than that achieved with delivery of the BMP via paracrine effects alone.

21. In view of the reasons and the facts described above, one skilled in the art would not be able to predict the organized, functional bone induction at a site of bone infirmity produced via implantation of *ex- vivo* transformed/transduced MSCs with BMP-2, as claimed in the subject Application.

The undersigned further declares that all statements made herein of his own knowledge are true, and that all statements made on information and belief are

believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Edward Schwarz

Date: October 19, 2006

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Maturation and Lineage-Specific Expression of the Coxsackie and Adenovirus Receptor in Hematopoietic Cells

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Key Words. *Adenovirus · Gene transfer · Hematopoietic cells · Coxsackie adenovirus receptor*

ABSTRACT

Adenovirus vectors have been used to transfer genes into both hematopoietic progenitor cells and tumor cells, including carcinoma cells that have metastasized to bone marrow (BM). However, the relative susceptibility of different subsets of hematopoietic cells is unknown. In permissive cells adenoviral-mediated gene transfer is mediated by the coxsackievirus and adenovirus receptor (CAR) protein and α_v integrins expressed on the cell surface of the target cells. This prompted us to investigate the expression of CAR on subpopulations of hematopoietic cells, determine whether this protein played a role in adenovirus-mediated gene transfer of hematopoietic cells and whether we could modulate CAR to enhance gene transfer efficiency. In this report we show that CAR is expressed on approximately 40% of all human BM cells, including erythroid and myeloid cells, but not lymphoid cells. Of the CD34$^+$ cells, 10%–15% expressed CAR, but this did not include most colony-forming progenitor cells, nor the most primitive CD38$^-$ subpopulation. The presence of CAR correlated well with gene transfer efficiency, but we were unable to induce CAR expression on immature, noncommitted progenitor cells. In conclusion, our results show that primitive hematopoietic progenitor cells lack CAR expression, but that expression is acquired during erythroid and myeloid differentiation. *Stem Cells 2000;18:176-182*

INTRODUCTION

The easy accessibility of hematopoietic progenitor cells and their ability to generate long-term progeny in vivo are two characteristics that make these cells important targets for gene therapy. For this purpose, a wide variety of viruses have been used including retro-, adeno-, adeno-associated, and lentiviruses [1-6]. Adenoviruses are able to infect noncycling cells and can be concentrated to extremely high titers; however, gene expression is transient. Thus, for gene therapy applications in which transient gene expression is desired, adenovirus may be the preferred vector for gene delivery into quiescent hematopoietic progenitor cells. Examples are the delivery of the amphotropic retroviral receptor or a mitogen to increase the sensitivity of cells to subsequent retroviral infection or improve the success rate of integration of a retrovirus-encoded transgene into the genome, respectively [7, 8]. The susceptibility of CD34$^+$ hematopoietic progenitor cells to adenovirus is somewhat controversial. Recent studies suggest that adenovirus vectors carrying a "suicide" gene may be suitable for bone marrow (BM) purging of cancer cells; in these experiments the breast carcinoma cells tested were much more easily transduced than freshly isolated BM cells, which were relatively resistant [9, 10]. Because of interest in using adenovirus as a purging vector, it is extremely important to establish the susceptibility of primitive BM cells to infection and the different mechanisms by which virus may enter the cell.

Cellular infection by adenovirus is a multistep process that involves the interaction of the trimeric fiber protein and the pentameric penton base protein of the virus with specific receptors on the target cells. First, the virus attaches to the cell, a process mediated by the fiber protein. The cellular

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receptor for the fiber protein was recently identified with the isolation of the common coxsackie and adenovirus receptor (CAR) protein [11]. After attachment, virus internalization and membrane permeabilization occur through the interaction of the penton base protein with α_v integrins on the target cells [12]. Although adenovirus infection is most efficient when both CAR and α_v integrins are present on the target cells, there is increasing evidence for successful adenovirus-mediated gene transfer using alternative pathways that circumvent the lack of either type of receptor [13, 14].

Adenovirus infection of human $CD34^+$ hematopoietic progenitor cells, a population that includes long-term repopulating stem cells, requires certain culture conditions and a high multiplicity of infectious (MOI) particles per cell [2]. Compared to certain primary cells or tumor cell lines, the relatively inefficient adenovirus-mediated gene transfer of hematopoietic progenitor cells may be in part due to the lack of expression of α_v integrins on their cell surface [15, 16]. Little is known about the expression of CAR on hematopoietic cells, although mRNA for the CAR protein has been demonstrated in $CD34^+$ cells isolated from leukopheresis products [17]. In light of these findings, we wanted to investigate whether the CAR protein is expressed on the cell surface of subpopulations of hematopoietic cells. If so, we were interested in answering the following questions: A) does the expression of the CAR protein correlate with susceptibility to adenoviral gene transfer in hematopoietic cells and B) can we identify cytokines that modulate the expression of CAR and therefore the gene transfer efficiency? We show that CAR expression on freshly isolated BM cells is mainly found on differentiated erythroid and myeloid cells, on a small proportion of $CD34^+$ progenitor cells, but not on lymphoid cells. Gene delivery into freshly isolated $CD34^+$ cells correlates well with the level of CAR expression, but still requires large amounts of virus.

Materials and Methods

Preparation of Human BM Cells

Discarded bags and attached filters from BM harvests of normal donors were rinsed with Iscove's modified Dulbecco's medium (IMDM) (Life Technologies; Grand Island, NY; http://www.lifetech.com) containing 2% fetal bovine serum ([FBS] Sigma Chemical Co.; St. Louis, MO; http://www.sigma-aldrich.com) to obtain the remaining BM cells. The cells were then centrifuged over a layer of Histopaque®-1077 (Sigma) to deplete erythroid and granulocytic cells. The cells were frozen (in IMDM, 50% FBS, 10% dimethylsulfoxide [Sigma]) and further separated on the day of the experiment. $CD34^+$ progenitor cells were enriched using a positive selection method as recommended by the manufacturer (Ceprate LC separation system, CellPro; Bothell, WA).

Cell Staining and Sorting

Flow cytometric detection of CAR on the cell surface was performed using the monoclonal antibody (mAb) RmcB [18], which was either directly conjugated to fluorescein isothiocyanate (FITC) or phycocrythrin. To define the different hematopoietic subpopulations and their expression of CAR, BM cells were simultaneously stained with anti-CD34-cyanine 5 (Becton Dickinson; San Jose, CA; http://www.bd.com), RmcB-FITC, and a mAb directed against one of the following lineage markers: CD33, CD14, or CD38 (Becton Dickinson), glycophorin-A, CD4 together with CD8, or CD19 (PharMingen; San Diego, CA; http://www.pharmingen.com). In every experiment irrelevant isotype controlled mAbs were used to determine background staining. All staining procedures were done in phosphate buffered saline ([PBS] Life Technologies) that contained 2% FBS. The cell labeling was performed on ice (35 min) after which the cells were washed twice. Propidium iodide ([PI] Sigma) (2 μg/ml) was added during the second wash prior to resuspension in PBS, 2% FBS. Three-color flow cytometric analysis and cell sorting were performed on a Coulter Epics® Elite ESP (Coulter, Hialeah, FL; http://beckmancoulter.com).

To analyze individual colonies for CAR expression, colonies were plucked from methylcellulose (MC), incubated for 1 h in PBS containing 2% FCS, to allow the MC to dissolve, spun down once and subsequently stained with the appropriate mAbs. Two-color fluorescence-activated cell sorter (FACS) analysis of the MC colonies and the suspension cultures (see later) were analyzed on a single laser FACScan (Becton Dickinson; Mountain View, CA).

Colony-Forming Cell (CFC) Assay

To determine the CFC content of the sorted $CD34^+$ BM cells, cells were plated in IMDM/0.9% MC media (Methocel MC, Fluka; Buchs, Switzerland; http://www.sigma-aldrich.com) containing 30% defined FBS (HyClone Laboratories Inc.; Logan, UT; http://www.hyclone.com) and the following human recombinant cytokines: *Steel* factor ([SF] 50 ng/ml), interleukin 3 ([IL-3] 20 ng/ml), GM-CSF (20 ng/ml), and erythropoietin ([Epo] 3 U/ml). IL-3, IL-6, and GM-CSF were generous gifts from Genetics Institute (Cambridge, MA; http://www.genetics.com). SF and Epo were purchased from R&D Systems (Minneapolis, MN; http://www.rndsystems.com). Duplicates of 1,000 cells (or as otherwise indicated) per 35 mm dish were plated. Colonies were scored in situ after 14–20 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air using well-established criteria [19].

Serum-Free Suspension Cultures

Enriched CD34$^+$ BM cells were cultured in serum-free medium prepared as described previously [20]. Cells were initially cultured in 1 ml volumes in 24-well culture plates and kept at a density below 1×10^6 cells/ml. The medium was supplemented with various combinations of the following cytokines: SF (50 ng/ml), Flt-3 ligand ([FL] 100 ng/ml), IL-6 (10 ng/ml), IL-3 (20 ng/ml), IL-11 (25 ng/ml), GM-CSF (20 ng/ml), and Epo (2 U/ml). FL was kindly provided by Immunex (Seattle, WA; http://www.immunex.com) and IL-11 by Genetics Institute. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. At subsequent days, the cultures were harvested, viable cells (excluding trypan blue) were counted using a hemocytometer and phenotypic analysis was performed as described above.

Adenovirus Construction and Preparation

The adenovirus vector that contains the green fluorescent protein gene (AdGFP) was kindly provided by *Bob Carter* and *Richard Mulligan* (Howard Hughes Medical Institution, Children's Hospital, Boston, MA; http://www.hhmi.org), and was constructed by first subcloning the GFP cDNA into pAdlox, a shuttle vector that contains a single loxP site. This expression cassette was linearized and cotransfected into CRE8 cells with the ψ5 helper virus, which is an E1- and E3-deleted version of Ad5 that contains loxP sites flanking the packaging site. Recombination occurs between the two linear molecules at the loxP sites [21]. We then plaque-purified the virus and expanded it on 293 cells using standard techniques. Each virus inoculum was purified by a CsCl step gradient followed by a CsCl equilibrium gradient, dialyzed against a glycerol buffer and stored at −20°C.

Adenoviral Infection Protocol and Analysis

After a short culture period (~4 h) CD34$^+$-enriched cells were incubated with AdGFP for 20 h at 37°C, at a MOI of 500, or otherwise indicated. The incubations were done in serum-free medium, supplemented with SF, FL, IL-6, and Epo in 100-200 μl volumes in 96-well plates when $<10^5$ cells were to be infected, or in 1 ml cultures in 24-well plates when the cell number was between 10^5-10^6. The analysis by FACS for green fluorescence intensity as a measure for gene transfer was performed immediately after the 24 h of culture.

Results

Expression of CAR on the Cell Surface of Subpopulations of Hematopoietic Cells

BM cell suspensions were stained with mAbs directed against CAR and various lineage markers representative for erythroid (glycophorin-A), myeloid (CD33 and CD14), and lymphoid (CD19 and CD4/CD8) cells. Figure 1 shows representative FACS profiles: CAR is expressed on ~40% of total BM cells, including glycophorin-A$^+$ cells, CD14$^+$, and CD33$^+$ cells. In contrast, very few CAR$^+$ cells can be demonstrated among the lymphoid CD19$^+$ or CD4/8$^+$ cells. To identify CAR expression on more primitive hematopoietic progenitor cells, BM cells were stained with a cocktail of mAbs identifying CD34, CD38, and CAR. From Figure 2A it is immediately clear that the level of CAR expression on CD34$^+$ cells is considerably lower than that on mature myeloid or erythroid cells (Fig. 1). Only 10%-15% of CD34$^+$ cells express CAR at a level comparable to that of, e.g., CAR$^+$CD33$^+$ cells (box 2, Fig. 2A) and 1%-2% express high levels of CAR (box 1, Fig. 2A). Counterstaining CD34$^+$CAR$^+$ cells with a combination of lineage markers (glycophorin-A, CD14, CD33, CD38, CD19, CD4, and CD8) revealed that these cells expressed one or more of these markers (data not shown). This finding suggests that CAR expression on hematopoietic BM cells may be limited mostly to mature erythroid and

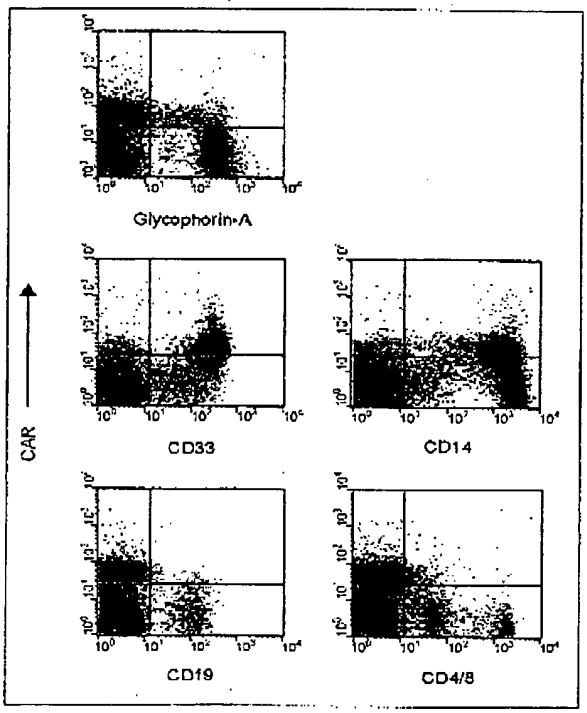

Figure 1. CAR is expressed on the cell surface of erythroid and myeloid, but not lymphoid cells. Depicted are representative profiles of live (PI$^-$) unseparated BM mononuclear cells.

myeloid cells and a small proportion of committed progenitor cells. Indeed, when CAR expression was determined on $CD34^+CD38^-$ cells, a population of cells that contains primitive nonobese diabetic/severe combined immunodeficiency (NOD/SCID) repopulating cells [22, 23], CAR expression was not detectable (Figs. 2B and 2C).

CAR Expression on CFC

The phenotypic analysis suggested that the majority of the $CD34^+$ progenitor cells does not express CAR. We wanted to investigate whether functional analysis could validate this result. $CD34^+$ BM cells were separated on the basis of CAR expression as indicated in Figure 2A (box 1-3) and the different subsets were then analyzed for their ability to form colonies in MC. One such analysis is shown in Table 1. Most of the colonies are recovered in the CAR^- fraction, a distribution that is in accordance with the relative CAR expression on $CD34^+$ cells.

Since the progeny of CFC are more differentiated cells, we were interested to determine the CAR expression on these cells. Individual colonies from the CAR^- fraction were therefore isolated and the cells stained with an anti-CAR mAb together with the appropriate lineage marker to confirm the morphological appearance of the colony. Colonies scored as BFU-E were counterstained with glycophorin-A and colonies

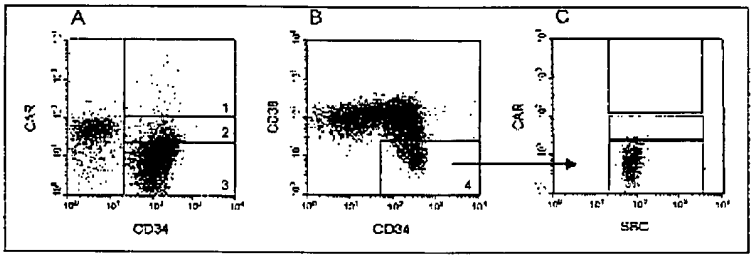

Figure 2. $CD34^+CD38^-$ progenitor cells do not express CAR. $CD34^+$ selected cells were stained with mAb directed against the indicated cell surface antigens. Shown are representative FACS profiles from live (PI^-) cells. A. In this experiment 11.4% of all $CD34^+$ cells expressed intermediate levels of CAR (box 2) and 1.2% high levels (box 1). B. Simultaneous staining with antiCD34 and antiCD38 mAbs revealed that the $CD34^+CD38^-$ cells (indicated by the cells in box 4) do not express CAR (C).

Table 1. The majority of CFC does not express CAR

[a]CD34+ subset	Fraction of all CD34+ cells	Number of Colonies (per 10^4 cells)			[b]Recovery of Colonies (%)		
	(%)	BFU-E	G/M/GM	GEMM	BFU-E	G/M/GM	GEMM
CAR^-	85.1	600	450	110	83	78	93
CAR^{int}	11.4	660	750	50	12	18	6
CAR^+	3.5	767	600	33	4	4	1

Data represent one of two experiments; the other experiment showed very similar results.

[a]$CD34^+$ cells were sorted as indicated in Figure 2A (boxes 1-3). Duplicates of 1,000 cells per dish were plated.

[b]The recovery was calculated as follows: for each subset and type of colony, the observed number per 10^4 cells was multiplied by the respective fraction that the subset represented of the total $CD34^+$ cells. This corrected number was then divided by the total number of colonies recovered (i.e., the sum of the corrected colony numbers of the three subsets) and multiplied by 100.

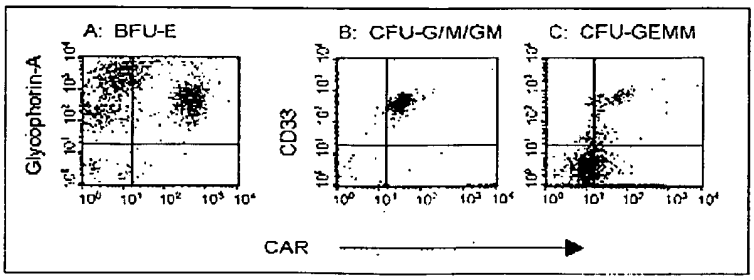

Figure 3. CAR^+ cells can be found among the progeny of all types of CFC. Shown are representative FACS profiles of live (PI^-) cells obtained by plucking MC colonies 14 days after the cells had been plated. Erythroid colonies (A) mostly showed a profile as presented here, but occasionally a level of CAR that was comparable to that of cells obtained from CFU-G/M/GM colonies (B) was detected. (C) Mixed lineage colonies contained cells from the erythroid, myeloid and megakaryocytic lineage.

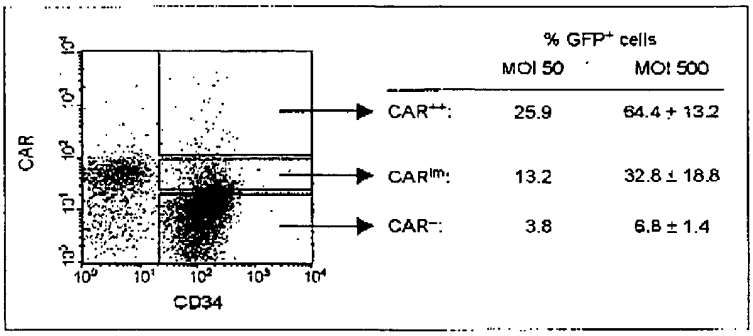

Figure 4. Expression of CAR correlates with adenovirus-mediated gene transfer efficiency. *Pre-enriched CD34⁺ cells were simultaneously stained with anti-CAR and anti-CD34 mAbs. CD34⁺ cells were sorted on the basis of CAR as indicated by the boxes. The results of 20 h exposure to AdGFP during a 24-h culture period for each fraction are shown in the figure. Each data point with MOI 500 consists of two to three independent experiments. The data points obtained with MOI 50 represent a single experiment. CAR^im cells are CD34⁺ cells that express intermediate levels of CAR. im = intermediate.*

scored as granulocytic and/or monocytic (CFU-G/M/GM) were counterstained with CD33. The multilineage colonies (CFU-GEMM) were also stained with CD33 to identify the myeloid component in an often dominant erythroid appearance. Figure 3 shows the various types of colonies that were identified by flow cytometry. A large proportion of cells isolated from erythroid colonies stained brightly positive for CAR (Fig. 3A). Cells from myeloid colonies all stained intermediate positive for CAR (Fig. 3B), as did the CFU-GEMM, but the level of CAR expression was on average lower than that of the myeloid colonies (Fig. 3C). Thus, while the majority of clonogenic progenitors is CAR⁻ (Table 1), their progeny show an increase in the level of CAR expression (Fig. 3).

Adenoviral Gene Transfer Efficiency in Relation to CAR Expression

To determine whether there was a correlation between CAR expression and efficiency of adenoviral gene transfer in CD34⁺ cells, CD34⁺ cells were separated on the basis of CAR expression, and the different fractions were cultured for 24 h. During the last 20 h of culture, cells were exposed to an adenovirus construct that contained the gene for the AdGFP. After 24 h, the culture was then analyzed by FACS for GFP expression (i.e., green fluorescence intensity). Figure 4 shows the combined results of three such experiments. The best gene transfer efficiency was indeed obtained with cells that expressed the highest level of CAR (CAR^++ cells); 64.4 ± 13.2%, compared to 6.8 ± 1.4% in cells that did not express CAR (CAR⁻ cells). The cells that expressed intermediate levels of CAR (CAR^im cells) showed intermediate levels of gene transfer: 32.8 ± 18.8%. This effect was dose-dependent; decreasing the MOI 10-fold reduced the gene transfer rate considerably. Thus, the level of CAR expression on freshly isolated CD34⁺ BM cells correlates well with the proportion of GFP⁺ cells after a 24-h exposure to AdGFP.

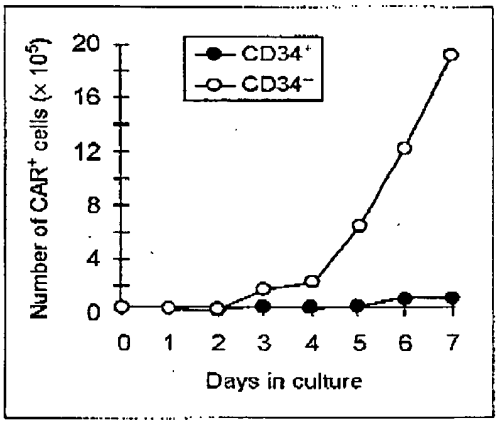

Figure 5. CAR⁺ cells produced in culture are mostly CD34⁻. *Presented is one of three experiments, showing the number of CAR⁺ cells (CD34⁺ and CD34⁻) that initiated the culture at day 0 and the production of CAR⁺ cells (CD34⁺ and CD34⁻) at subsequent days. The number of cells was calculated by multiplying the total cell number by the fraction of cells of a particular phenotype obtained by FACS analysis.*

Cytokines Do Not Induce CAR Expression

BM cells enriched for CD34⁺ progenitor cells were cultured under serum-free conditions to determine whether one or a combination of cytokines could induce CAR expression on such cells. The following cytokines were tested in one, two, and four-day cultures, either alone or in combination: SF, Flt-3, IL-6, IL-3, IL-11, GM-CSF, and Epo. No cytokine or combination thereof—33 conditions were tested—could be identified that showed a superior effect on CAR expression (data not shown). All subsequent cell cultures were therefore performed in serum-free medium, supplemented with SF, FL, IL-6, and Epo, a culture condition demonstrated to maintain the most

primitive hematopoietic cells [1, 24, 25]. Over a seven-day culture period, the number of $CD34^+$ cells that express CAR stays nearly constant (Fig. 5). However, the number of CD34 cells that express CAR increases dramatically with time. Thus, as $CD34^+$ cells lose CD34 expression, they acquire CAR. This result, together with the phenotypic analysis and functional CFC data, suggest that expression with CAR in hematopoietic cells is related to myeloid and erythroid differentiation.

Discussion

Adenovirus-mediated gene transfer is highly efficient in permissive cells, such as HeLa cells, or nonpermissive cells stably infected with the gene encoding CAR [11, 14]. In contrast, we show here that the transduction of genes into primitive $CD34^+$ hematopoietic cells by an adenovirus construct is not very effective. In our hands, only 15%-20% of $CD34^+$ cells exposed for 20 h to adenovirus were transduced, a result that directly correlated with the level of CAR expressed on the cell surface. However, CAR expression was found to be associated with cellular differentiation. These results predict very low adenovirus-mediated gene transfer into immature long-term repopulating hematopoietic stem cells (HSCs). Indeed, in one experiment in which purified $CD34^+CD38^-$ cells were exposed to AdGFP for 20 h of the 24 h in culture, only 2% gene transfer efficiency could be demonstrated (data not shown). These results appear to contrast with a previous report showing that quiescent $CD34^+CD38^-$ cells were GFP^+ after exposure to an adenovirus GFP construct [2]. Several reasons may account for this difference: first, the post-infection time allowing for gene expression (24 h in our experiment versus 48 h), and second, the starting population that was infected. We infected purified $CD34^+CD38^-$ cells, whereas *Neering et al.* used total $CD34^+$ cells and analyzed the proportion of transfected $CD34^+CD38^-$ cells by FACS. It is possible that there are accessory cells present in the $CD34^+$ cell population that facilitate gene transfer into other cells. The mechanism by which this occurs is unclear, but it is tempting to speculate that these cells produce certain cytokines that upregulate cell surface molecules, as yet unidentified, that are important for adenoviral infections in primitive hematopoietic cells. Interestingly, although adenoviral gene transfer into human primitive cells is inefficient at best, murine long-term repopulating HSCs are quite efficiently transducible with the same construct (unpublished data, 1999).

Several approaches have been taken to improve adenovirus infection of otherwise nonpermissive cells, such as modulating the viral surface structures with which the virus may attach to the target cells and the use of agents to facilitate the virus-target cell binding [26-28]. Our approach, i.e., trying to induce CAR expression on hematopoietic progenitor cells, has so far been unsuccessful. The cytokines we tested were chosen based on previous studies describing their (relative) beneficial effect on $CD34^+$ cells in maintaining NOD/SCID mouse repopulating ability [1, 29, 30]. Because our goal was not only to induce CAR, but also to maintain phenotype/function, the cytokines that were tested were limited to SF, Flt-3, IL-6, IL-3, IL-11, GM-CSF, and Epo. However, studies with other cell types may point us towards examining other cytokines, not usually thought of in relation to culturing $CD34^+$ cells. In this regard it is of interest that IL-2 was found to induce the expression of the fiber receptor on the cell surface of lymphocytes, whereas this receptor was undetectable in nonculturned, freshly isolated lymphocytes [31]. Whether this receptor was indeed CAR needs to be verified, or, for example the $\alpha_M\beta_2$ integrin, as others have shown to be involved in adenovirus binding to human monocytic cells [32]. Interestingly, a small subset of $CD34^+$ cells do express this integrin [33] and may provide a tool to improve adenovirus-mediated gene transfer into hematopoietic progenitor cells.

In conclusion, our results clearly demonstrate that CAR is expressed on hematopoietic cells and that its expression is directly related to the susceptibility of these cells to adenoviral gene transfer. We also demonstrate that the majority of CAR^+ hematopoietic cells are lineage-committed cells and not the more primitive $CD34^+$ progenitor cells. Our findings, along with those of others [2], that high MOIs are needed to successfully infect hematopoietic cells with adenovirus, suggest that at low MOI, adenovirus may provide a good vehicle for oncolytic therapy in cancer.

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Stage-Specific Oligonucleotide Uptake in Murine Bone Marrow B-Cell Precursors

By Qiuyan Zhao, Thomas Waldschmidt, Eric Fisher, Charles J. Herrera, and Arthur M. Krieg

Fluorescein isothiocyanate (FITC)-conjugated phosphodiester and phosphorothioate oligonucleotides were used in four-color flow cytometry with murine bone marrow cells stained with monoclonal antibody specific for the differentiation markers B220, S7 (CD43), and BP-1 to show possible stage-specific oligonucleotide uptake. Relatively low uptake was observed among pre–Pro- and early Pro-B cells. Late Pro-B- and pre-B cells had increased oligonucleotide uptake, whereas B cells had a lower level. Cell membrane binding of oligonucleotides varied during B-cell differentiation in parallel with internalization, which was documented by confocal microscopy. An FITC-conjugated polyanionic dextran sulfate also showed differentiation-related B-cell association, suggesting the presence of cell membrane binding sites specific for polyanions as opposed to a unique feature of the DNA backbone. Interpretation of antisense experiments in murine bone marrow cells will need to account for the heterogeneous oligonucleotide uptake among differentiating B cells.

ANTISENSE oligodeoxynucleotides (ODNs) have become a widely used research tool. By blocking gene translation in a sequence-specific manner, antisense ODNs can provide very useful information on the function of genes of interest. Originally exploited solely for the experimental regulation of gene expression in studies of cultured cell lines, antisense ODNs have been increasingly applied to investigations using primary cell cultures or in vivo administration.[1,2] Despite these rapid strides in developing applications for antisense ODNs, many of the relevant basic mechanisms remain poorly understood.

For an antisense oligonucleotide to have an effect, it must first enter the target cell. ODN uptake in cell lines is saturable, sequence independent, and temperature and energy dependent.[3] Although there is some evidence to suggest that such uptake may occur through an 80-kD membrane protein,[4,5] the gene for this has not yet been cloned or further characterized. Unfortunately, the mechanism(s) of ODN uptake into primary cells has not been investigated as thoroughly as in cell lines.

Antisense ODNs have been used by several research groups investigating the role of various oncogenes in hematopoiesis and mitogenesis.[6-14] Such studies have generally been performed in cell lines. It would be of great interest to study hematopoiesis and B-cell differentiation in primary cells using antisense ODNs. If ODN uptake were heterogeneous among different subsets of primary cells, the interpretation of these experiments could be altered. Indeed, we have previously shown that, although oligonucleotide uptake in murine spleen cells was saturable and temperature dependent, like uptake in cell lines, this uptake was quite heterogeneous: cultured B cells had much higher uptake than T cells.[15]

One of the most popular methods for studying the cellular uptake of oligonucleotides has been the use of radiolabeled oligonucleotides. Unfortunately, the results of experiments using end-labeled oligonucleotides can be affected by cellular phosphatases.[16,17] Another important caveat to the interpretation of studies using radiolabeled oligonucleotides is that dead cells can have much higher oligonucleotide uptake than live cells.[18-20] Thus, differences in the uptake of radiolabeled oligonucleotides between different cell populations could result from variations in the proportion of dead cells unless this has been carefully controlled. A further limitation of using radiolabeled oligonucleotides for studies of heterogeneous cell populations (such as primary cells) is that this technique does not allow one to readily distinguish whether uptake results from many cells taking up a modest amount of oligonucleotide or from a cell subpopulation taking up a great deal.

Flow cytometry offers an attractive alternative to radiolabeled oligonucleotides for determining whether oligonucleotide uptake in primary cells is heterogeneous or uniform. Using a two-laser cytometer, cells can be cultured with fluorescein isothiocyanate (FITC)-conjugated oligonucleotides and then stained with three different cell subset-specific monoclonal antibodies (MoAbs) conjugated to three different dyes fluorescing at distinct wavelengths. Data are analyzed by gating on the desired combination of antibody staining characteristics to determine the relative levels of oligonucleotide uptake in each cell subpopulation of interest.

Progenitor bone marrow (BM) cells committed to the B-cell lineage, all of which express the B220 marker, can be readily studied using flow cytometry. The pre–Pro-B, early and late Pro-B, Pre-B, and B-cell stages can be distinguished by their differential expression of several cell surface molecules, including heat-stable antigen, and determinants recognized by S7 and BP-1.[21] The S7 MoAb recognizes CD43 (referred to as S7 herein), which is expressed in B-cell precursors up to the Pre-B cell stage of differentiation[21] and is also expressed in most myeloid cells. The BP-1 MoAb detects a cell surface protein (termed BP-1 herein) that is expressed on the large proliferating late pro–B- and pre–B-cell populations but not on earlier or later B-cell precursors.[21,22] In the present study, we used four-color flow cytom-

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etry with FITC-oligonucleotides and antibodies to these determinants to investigate whether oligonucleotide uptake may be a stage-specific marker of B-cell differentiation. We also studied the temperature and oligonucleotide backbone dependence of uptake by murine BM B-cell progenitors.

MATERIALS AND METHODS

Mice. DBA/2 mice were bred and housed in the University of Iowa specific pathogen-free animal care unit with free access to food and water. NZB mice were purchased from The Jackson Laboratory (Bar Harbor, ME). At 6 to 8 weeks of age, they were killed by cervical dislocation and BM cells were prepared sterilely for further study.

Oligonucleotides. FITC was conjugated to the oligonucleotides through the 5'-hydroxyl using a fluorescein amidite (Pharmacia, Piscataway NJ). In the text, FITC-O-oligonucleotide refers to phosphodiester oligonucleotides bearing a 5' fluorescein and FITC-S-oligonucleotide refers to phosphorothioates bearing a 5' fluorescein. Oligonucleotides were synthesized on an Applied Biosystems Inc (Foster City, CA) model 394 DNA synthesizer. Phosphodiester oligonucleotides were synthesized using standard β-cyanoethyl phosphoramidite chemistry. Phosphorothioate linkages were introduced by oxidizing the phosphite linkage with elemental sulfur instead of the standard iodine oxidation.[23] Oligonucleotides were deprotected by treatment with concentrated ammonia at 55°C for 12 hours and were purified by gel exclusion chromatography and polyacrylamide gel electrophoresis and lyophilized to dryness before use. The ratio of absorption at 490 nm and 260 nm was identical for the different oligonucleotides, indicating that they were equivalently pure. Heteropolymer oligonucleotides conjugated to Cy3.18 were synthesized as described.[24] Each modified FITC-oligonucleotide was synthesized with two different sequences (5' GAGAACGCTGGACCT-TCCAT 3' and 5' TGCTAGCTGTGCCTGTACCT 3'). Because there was no apparent difference in cellular uptake in initial experiments, these two sequences were used interchangeably in subsequent experiments.

Cell cultures. Murine BM cell cultures were set up by preparing single-cell suspensions, washing twice, and culturing 5×10^5 cells in 150l RPMI-1640 media containing 10% heat-inactivated fetal bovine serum, 50 μmol/L 2-ME, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine at 37°C, 5% CO_2, and 100% humidity. Where indicated, 1 μg of FITC-oligonucleotide or FITC-dextran was added at the beginning of culture. Dead cells were eliminated by purification over lymphocyte M (Accurate Chemical Co, Westbury, NY) before analysis.

Antibodies and reagents. Phycoerythrin (PE)-6B2 was purchased from Pharmingen (San Diego, CA). Texas Red-avidin (used to detect biotin MoAb) was purchased from Vector Labs (Burlingame, CA). Antibody biotinylation was performed using standard techniques. Cyanine-5 conjugated antibodies were prepared using a commercial kit from Biological Detection Systems (Pittsburgh, PA) according to the manufacturers instructions. Polyanionic FITC-dextran (molecular weight [MW] 10,000) was purchased from Molecular Probes (Eugene, OR). PE-avidin was purchased from Leinco (St Louis, MO).

Flow cytometry. Cells for flow cytometry were washed and stained in Hank's balanced salt solution (HBSS) with 0.01% sodium azide with the indicated antibodies for 30 minutes at 4°C using 1 μg 24G2 (anti-FcR) and normal rabbit serum to block nonspecific binding. Data on viable cells (30,000 per sample) were collected by four-color flow cytometry on an EPICS 753 (Coulter, Hialeah, FL) equipped with a 3-decade log amplifier and two 400-mW lasers: a 488-nm argon-ion laser and a 600-nm Rhodamine 6G dyehead laser.

Table 1. Time Course of Oligonucleotide Uptake and Egress

Time for Uptake	Mean FITC Fluorescence* B220+, BP-1+	B220+, BP-1−
Autofluorescence	5.5	3.4
0.5 h	14.5	12.9
1.5 h	41.8	29.8
3 h	65.9	38.6
5 h	61.5	37.2
12 h	54.3	32.5
24 h	63.1	38.4
48 h	75.4	46.8
1.5 h efflux	45.5	30.4
3 h efflux	32.4	24.0

* Mean FITC fluorescence was converted from channel number. Bone marrow cells were set up at 37°C in culture essentially as described in the Materials and Methods, except that 8×10^5 cells were cultured in 0.5 mL medium for 48 hours. Three micrograms of FITC-S-oligonucleotide (final concentration, 1 μmol/L) was added to each sample at the appropriate time after initiation of culture to allow uptake for the times shown. To study efflux, cells were cultured with FITC-S-oligonucleotide for 12 hours, washed, and then cultured at 37°C in medium without oligonucleotide for the times indicated. All samples were then washed and stained together with biotin-BP-1 and Cyanine-5-6B2 followed by PE-avidin. Propidium iodide was added immediately before analysis and the data were gated on B220+, PI− cells.

Data were analyzed using Coulter Elite software after gating on living lymphocytes. All experiments shown were performed at least three times with similar results. For experiments using all four colors, "living lymphocytes" were defined by forward versus orthogonal gates. Some experiments were performed in which viability was confirmed by propidium iodide exclusion. Propidium iodide was evaluated and therefore, staining was limited to cyanine-5-BP-1 and PE-6B2 (see, eg, Table 1). This finding showed that the differential oligonucleotide uptake between the 6B2+ BP-1− and 6B2+ BP-1− populations did not result from variations in cell viability. Isotype control antibodies were used to determine nonspecific staining. Band pass filters were at 525 (FITC), 575 (PE), 635 (Texas red), and 670 (Cyanine 5). In some experiments, a FACScan (Becton Dickinson, Mountain View, CA) containing a 15-mW argon-ion laser was used with Lysis II software.

Confocal microscopy. Confocal microscopy was performed after cell culture as described above using a BioRad (Richmond, CA) MRC600, with a krypton/argon laser and Nikon (Melville, NY) optiphoto optics with a 60× 1.4 NA objective, and images were printed with a Sony UP5000 video printer. Cells were washed at 4°C but not fixed and were immediately analyzed under coverslips in HBSS. In some experiments, a temperature-controlled stage was used to maintain samples at 4°C.

RESULTS

Determination of B-cell progenitor gates. B220+ mouse BM B-cell progenitors can be divided into distinct maturational stages based on their surface staining for S7 and BP-1.[21] To distinguish these stages, gates were then set on the S7+ and S7− and the BP-1+ and BP-1− subsets of the B220+ population (Fig 1A and B). As reported by Hardy et al,[21] the B220+S7+ had the lowest levels of B220 and were less

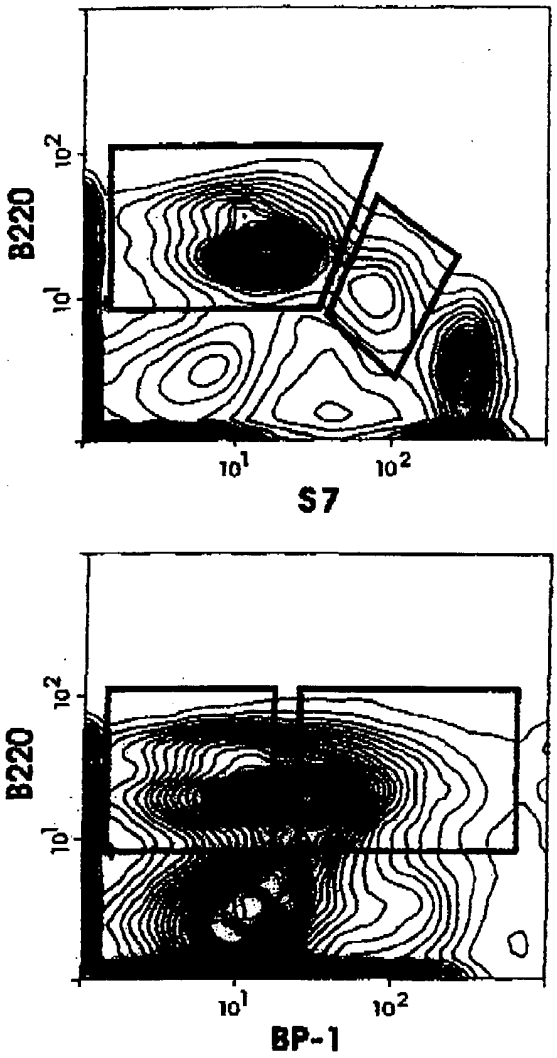

Fig 1. Setting of progenitor B-cell gates. Fresh DBA/2 BM cells were stained for flow cytometry as described with PE-6B2 (B220), cyanine-5-S7, and biotin-BP-1. Forward versus orthogonal gates were set to include greater than 95% of the B220$^+$ population. Two-color contour plots are shown to indicate how gates were set on the B220$^+$S7$^+$, B220$^+$S7$^-$, B220$^+$BP-1$^+$, and B220$^+$BP-1$^-$ cells. These gates were then combined to define the B220$^+$S7$^+$BP-1$^-$, B220$^+$S7$^+$BP-1$^+$, B220$^+$S7$^-$BP-1$^+$, and B220$^+$S7$^-$BP-1$^-$ cell subsets. Because the forward versus orthogonal scatter was set to include large cells, myeloid cells, which are S7$^+$, B220$^-$, are readily apparent. Myeloid cells have increased autofluorescence, accounting for their apparent dull positivity for BP-1.

common than B220$^+$S7$^-$ cells. Combinations of the appropriate gates could thus distinguish pre–pro- and early pro-B cells (B220$^+$S7$^+$BP-1$^-$) from late pro-B cells (B220$^+$S7$^+$BP-1$^+$), pre-B cells (B220$^+$S7$^-$BP-1$^+$), and B cells (B220$^+$S7$^-$BP-1$^-$).[21] In separate experiments, the validity of this gating and the consistency of our staining with that of Hardy et al[21] was confirmed using additional MoAb including M1/69 (heat-stable antigen) and sIg (not shown). Early pro-B cells and pre–pro-B cells, which are both B220$^+$S7$^+$BP-1$^-$, can be distinguished by their staining for heat-stable antigen.[21] These populations had no substantial difference in oligonucleotide uptake, and so are shown together in the figures as pre-pro-B.

Cellular association of oligonucleotide in progenitor B cells is stage specific. BM cells were cultured with FITC-oligonucleotides as described in Materials and Methods, washed, and stained as described above to enable gating on four stages of B-cell differentiation. FITC histograms of these gated populations showed relatively low oligonucleotide uptake in the pre–pro-B- and B-cell populations, but higher uptake in the pro-B- and pre-B-cell populations (Fig 2). Uptake was uniformly low among Thy1$^+$, B220$^-$ BM T-cell precursors (not shown). This differential uptake did not appear to be an artifact of the FITC tag on the oligonucleotide because it was also seen in separate experiments using oligonucleotides labeled with Cy 3.18, a fluorochrome with distinct physicochemical properties[24] (data not shown). At the 4-hour time point studied, there is relatively little degradation of the FITC-O-oligonucleotide[20] (data not shown).

Similar patterns of uptake were seen in nonautoimmune DBA/2 and autoimmune NZB mice (Fig 2). Slightly increased uptake was present in the most mature subset of NZB BM B cells, which may be caused by the fact that NZB B-cell precursors show increased spontaneous activation[25] and that oligonucleotide uptake is increased among activated cells.[15]

Temperature and backbone dependence of oligonucleotide uptake. To investigate the temperature dependence of oligonucleotide uptake, cells were cultured with oligonucleotide at 4°C and compared with those cultured at 37°C. FITC-oligonucleotide binding to cell membranes at 4°C was specific in all cell subsets because it was competed by unconjugated phosphorothioate competitor using previously described methods.[20] In addition, we compared the relative levels of uptake of FITC-O-and FITC-S-oligonucleotides and a polyanionic FITC-dextran of similar MW. The FITC-S-oligonucleotides showed substantially increased binding to all cell subsets compared with the unmodified backbone (Fig 3). The polyanionic dextran was studied because of our preliminary data indicating that it binds to the same cell surface sites as oligonucleotides and has similar intracellular localization by confocal microscopy (not shown). Cellular association of both oligonucleotides and the polyanionic dextran was temperature- and time-dependent (Fig 3).

Even in the cells cultured on ice, there was a modest but consistent increase in the level of oligonucleotide present in the late pro-B and pre-B cells compared with that in the other B-cell stages, suggesting that the former cells have

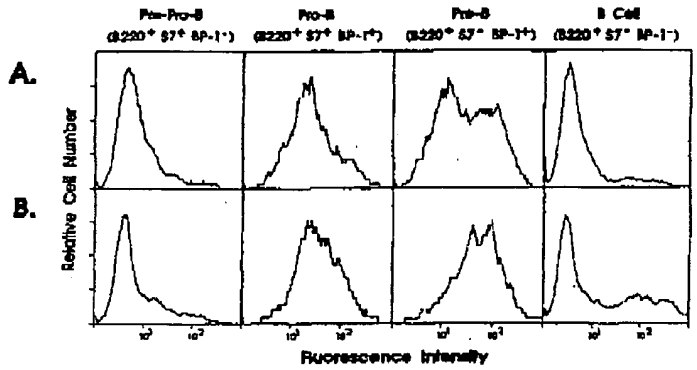

Fig 2. Stage-specific cellular association of oligonucleotide. DBA/2 (top panels) and NZB (bottom panels) BM cells were cultured for 4 hours at 37°C with FITC-O-oligonucleotide to allow uptake (see Materials and Methods) and then stained as in Fig 1. FITC histogram profiles are shown for the indicated cell subsets for each mouse strain.

higher levels of membrane oligonucleotide binding sites. Both oligonucleotides and the polyanionic dextran showed the same pattern of preferential association with the late pro-B and pre-B cells at both temperatures. These cells were slightly larger[26] and had slightly higher levels of autofluorescence than the pre–pro-B and B cells (compare "no oligo" histograms, Fig 3), but this was still below the level seen with oligonucleotides or dextran. By gating on small or large cells within each subset[15] and by examining the forward scatter profiles of the subsets, we verified that the differential uptake among the subclasses could not be attributed to differences in cell volume or surface area (data not shown). Although the oligonucleotides could readily compete the surface binding of the FITC-dextran, an unconjugated polyanionic dextran had little reproducible effect on FITC-O-oligonucleotide cell binding (data not shown).

Time course of oligonucleotide uptake and efflux. BM cells were cultured for 48 hours at 37°C and FITC-S-oligonucleotide was added at various time points. For efflux studies, the oligonucleotide was washed out the indicated number of hours before flow cytometry analysis (Table 1). Because the B220+ S7+ BP-1- and B220+ S7- BP-1- subsets had low oligonucleotide uptake and the B220+ S7+ BP-1+ and B220+ S7- BP-1+ subsets had high oligonucleotide uptake, those subsets were pooled into two groups: B220+, BP-1- and B220+, BP-1+ (Table 1). This pooling enabled us to use the detector that had been devoted to S7 for propidium iodide instead, thereby ensuring that the cells analyzed were viable. These studies confirmed that at every time point during FITC-S-oligonucleotide uptake and efflux, oligonucleotide levels were higher among the B220+, BP-1+ cells (Table 1). Uptake reached a plateau by 3 hours. Fluorescence microscopy of unwashed cells showed that cellular fluorescence was typically at least as intense as the background level in the medium containing FITC-oligonucleotide (not shown). Cell-associated oligonucleotide declined by approximately

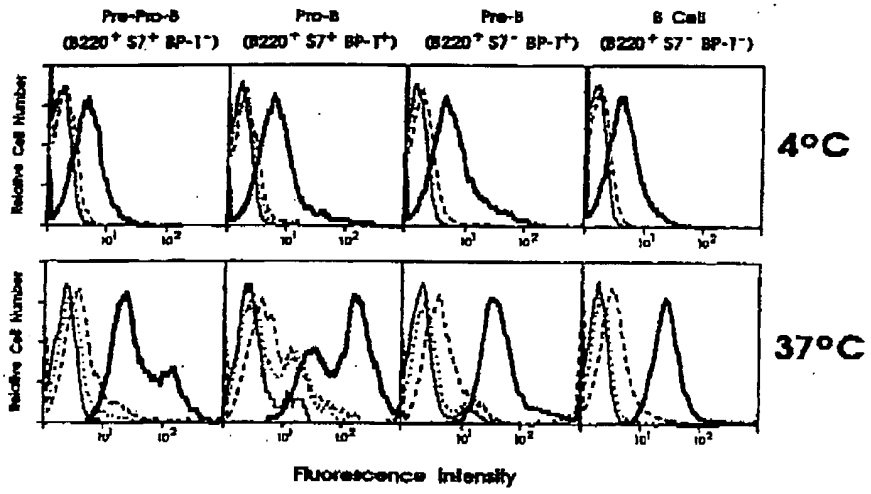

Fig 3. Temperature dependence of oligonucleotide uptake. DBA/2 BM cells were cultured as in Fig 2 either at 4°C (top) or 37°C (bottom) and then stained as above. FITC histogram profiles are shown for the indicated cell subsets in parallel cell aliquots cultured either with no oligonucleotide (—), FITC-O-oligonucleotide (- - -), FITC-S-oligonucleotide (——), or FITC-dextran (· · · ·).

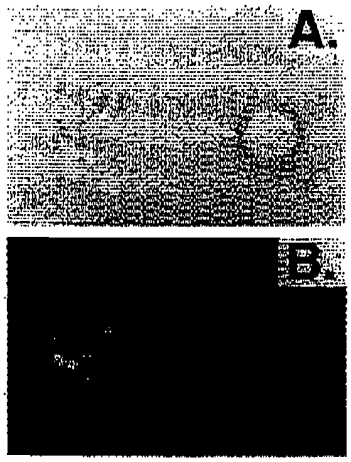

Fig 4. Intracellular oligonucleotide localization. Phase contrast (A) and fluorescence image (B) of typical BM cells obtained by confocal microscopy, showing the heterogeneity of uptake seen with an FITC-S-oligonucleotide. Although the cell on the right is slightly out of focus, no uptake was visible within that cell in any plane of focus.

50% in both cell subsets after 3 hours of efflux (Table 1). Uptake was more gradual in BM cells that had not been precultured for 48 hours (data not shown).

Intracellular oligonucleotide localization. The temperature dependence of cellular oligonucleotide association seen in the experiments above suggested the possibility that the fluorescence seen in cells cultured with oligonucleotide at 4°C was caused by binding to the cell surface and that cell culture at 37°C resulted in oligonucleotide internalization. To test this hypothesis, we used confocal microscopy. In cells cultured on ice, FITC-oligonucleotide was located solely on the cell membrane by confocal microscopy; in cells cultured at 37°C, oligonucleotide was present intracellularly (Fig 4 and data not shown). Autofluorescence was not detectable under the conditions used. Among cells that took up FITC-oligonucleotide, fluorescence was typically speckled and cytoplasmic with very little nuclear fluorescence (Fig 4). Both FITC-O-oligonucleotides and FITC-S-oligonucleotides showed similar patterns of localization, but the level of fluorescence was typically higher in cells cultured with the FITC-S-oligonucleotides.

DISCUSSION

The interpretation of experiments using antisense oligonucleotides in vivo or in primary cell cultures is critically dependent on whether cell uptake is homogeneous or heterogeneous. With this in mind, the present study was performed to determine the relationship, if any, between oligonucleotide uptake and murine BM cell differentiation.

As previously described by Hardy et al,[21,22] B-lineage lymphocytes pass through several sequential stages with distinct expression of cell surface molecules as they develop from their hematopoietic progenitors. Using flow cytometry, we could readily distinguish FITC-oligonucleotide uptake among pre–Pro- and early Pro-B cells ($B220^+S7^+BP\text{-}1^-$) from late Pro-B cells ($B220^+S7^+BP\text{-}1^+$), Pre-B cells ($B220^+S7^-BP\text{-}1^+$), and B cells ($B220^+S7^-BP\text{-}1^-$). We found that uptake was quite low among the pre–Pro- and early Pro-B cells, increased in the $BP\text{-}1^+$ late Pro-B- and Pre-B-cell populations, and was lower among mature B cells. By examining cell association of FITC-oligonucleotide in cells cultured at 4°C, we showed that the level of cell membrane oligonucleotide binding differed among cell subsets in the same way as cell association at 37°C.

Our analysis of the time course of FITC-S-oligonucleotide uptake into and efflux from murine BM cells showed that, in both the $BP\text{-}1^+$ subsets with relatively high uptake and the $BP\text{-}1^-$ subsets with low uptake, the levels of cell-associated oligonucleotide plateaued after culture at 37°C for 3 hours. Efflux was gradual, with approximately 50% decrease in cell-associated fluorescence by 3 hours. Previous studies of oligonucleotide uptake in various cell lines have found that a plateau in uptake is reached after from 1 hour[27] to more than 6 to 12 hours.[19,28-30] However, interpretation of some of these data using radiolabeled oligonucleotides is not straightforward (both for the reasons given in the introduction and because the oligonucleotides were not shown to be intact, leaving open the possibility that the label may have been released from degraded oligonucleotide and incorporated into cellular nucleic acids). As shown previously, FITC-phosphorothioate oligonucleotides are highly stable in cells,[20,31] thus avoiding these problems.

Confocal microscopy showed that oligonucleotide is internalized by living BM cells and shows a somewhat stippled predominantly cytoplasmic localization, compatible with endocytotic uptake. There was very little nuclear localization. This is similar to oligonucleotide distribution within spleen cells.[20,32] These studies are in agreement with those of other investigators conducted in cell lines using a variety of techniques.[3,4,28,33-37] Indeed, predominant nuclear localization is generally only reported in studies using microinjection of oligonucleotides or in dead cells.[38,39] From these and other unpublished studies, it appears increasingly likely that the rate-limiting step in antisense oligonucleotide efficacy may be an exit from the endosomes. Exit from the endosomes is likely followed by rapid nuclear uptake.[27,28,39] The oligonucleotide backbone appears to have little effect on this localization, although it can be dramatically altered by conjugation of lipophilic groups.[32,33]

Of note, the stages with the highest oligonucleotide uptake are the same as those with the highest rate of proliferation.[26] This may be related to our previous findings of markedly increased oligonucleotide uptake in mitogen-stimulated peripheral B cells.[13] A possible explanation for increased oligonucleotide uptake among proliferating cells is their need to obtain sufficient nucleic acids to double their DNA content.[35] This finding may explain why cell lines have generally been found to have higher rates of oligonucleotide uptake than do primary cell lines.

Oligonucleotide binding to cell membranes was specific in that it was competable. However, the cell membrane DNA